Involvement of a novel Tnf receptor homologue in hair follicle induction

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Although inductive interactions are known to be essential for specification of cell fate in many vertebrate tissues, the signals and receptors responsible for transmitting this information remain largely unidentified. Mice with mutations in the downless (*dl*) gene have defects in hair follicle induction, lack sweat glands and have malformed teeth[1]. These structures originate as ectodermal placodes, which invaginate into the underlying mesenchyme and differentiate to form specific organs[2–4]. Positional cloning of the *dl* gene began with identification of the transgenic family OVE1. One branch of the family, dl^{OVE1B}, carries an approximately 600-kb deletion at the *dl* locus caused by transgene integration. The mutated locus has been physically mapped in this family[5], and a 200-kb mouse YAC clone, YAC D9, has been identified and shown to rescue the *dl* phenotype in the spontaneous $dl^{Jackson}$ (dl^{J}, recessive) and Dl^{Sleek} (Dl^{slk}, dominant negative) mutants[6]. Here we report the positional cloning of the *dl* gene, which encodes a novel member of the tumour necrosis factor (Tnf) receptor (Tnfr) family. The mutant phenotype and *dl* expression pattern suggests that this gene encodes a receptor that specifies hair follicle fate. Its ligand is likely to be the product of the tabby (*Ta*) gene, as *Ta* mutants have a phenotype identical to that of *dl* (ref. 1) mutants and *Ta* encodes a Tnf-like protein.

We used cDNA selection to isolate expressed sequences from the *dl* locus. First, a BAC library was screened using primers derived from sequencing of YAC D9 subclones. Of the eight BACs identified, BAC 508K21 was found to overlap most with YAC D9 and was used as driver for cDNA selection. One of the selected cDNA fragments (c*DS446*, 390 bp) was expressed in embryonic epidermis and absent from the dl^{OVE1B} genome, suggesting that it is part of the *dl* transcript.

Screening of an embryonic day (E) 17.5 skin, foot and tail cDNA library with c*DS446* identified two positive clones, one of which was fully sequenced. We used RACE to extend the 5′ end. The extended cDNA has a 259-nt 5′ UTR with stop codons in all 3 reading frames, a 1,347-nt ORF with an ATG that is in the proper context for initiation of translation[7] and a 2,100-nt 3′ UTR.

The ORF encodes a protein predicted to consist of a cleavable amino-terminal signal peptide, a mature extracellular domain (159 aa), a single transmembrane region and an intracellular domain (237 aa; Fig. 1*a*). BLAST comparison of this protein sequence with non-redundant databases detected regions of similarity with members of the Tnfr family[8] (Fig. 1*b*,*c*). As the *dl* mutant phenotype is analogous to human hypohidrotic/anhidrotic ectodermal dysplasia (EDA), and the encoded protein

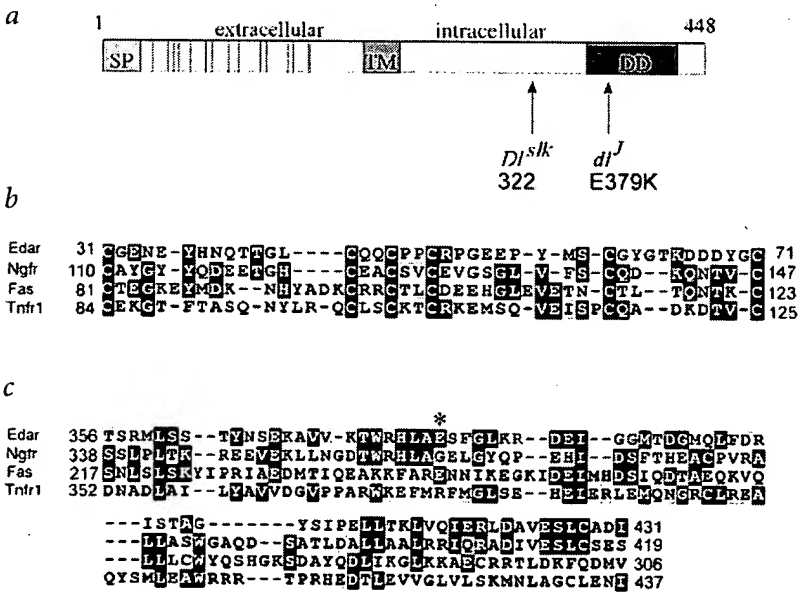

Fig. 1 Domain structure and amino acid alignments of Edar. ***a***, Predicted domains of Edar, showing the signal peptide (SP), Tnfr-like extracellular domain, transmembrane sequence (TM) and intracellular region including the putative death domain (DD). The extracellular cysteine residues are indicated by vertical lines. The positions of the dl^{J} and Dl^{slk} mutations are indicated. ***b***, The first cysteine-rich repeat of the mouse Edar extracellular sequence compared with similar extracellular sequences in the rat p75 nerve growth factor receptor (Ngfr), mouse Fas and mouse Tnfr1. Amino acid position is indicated by the numbering at the beginning and end of each sequence. ***c***, Comparison of Edar, rat Ngfr, mouse Fas and mouse Tnfr1 death domains. Glutamate 379, mutated in dl^{J}, is indicated by an asterisk.

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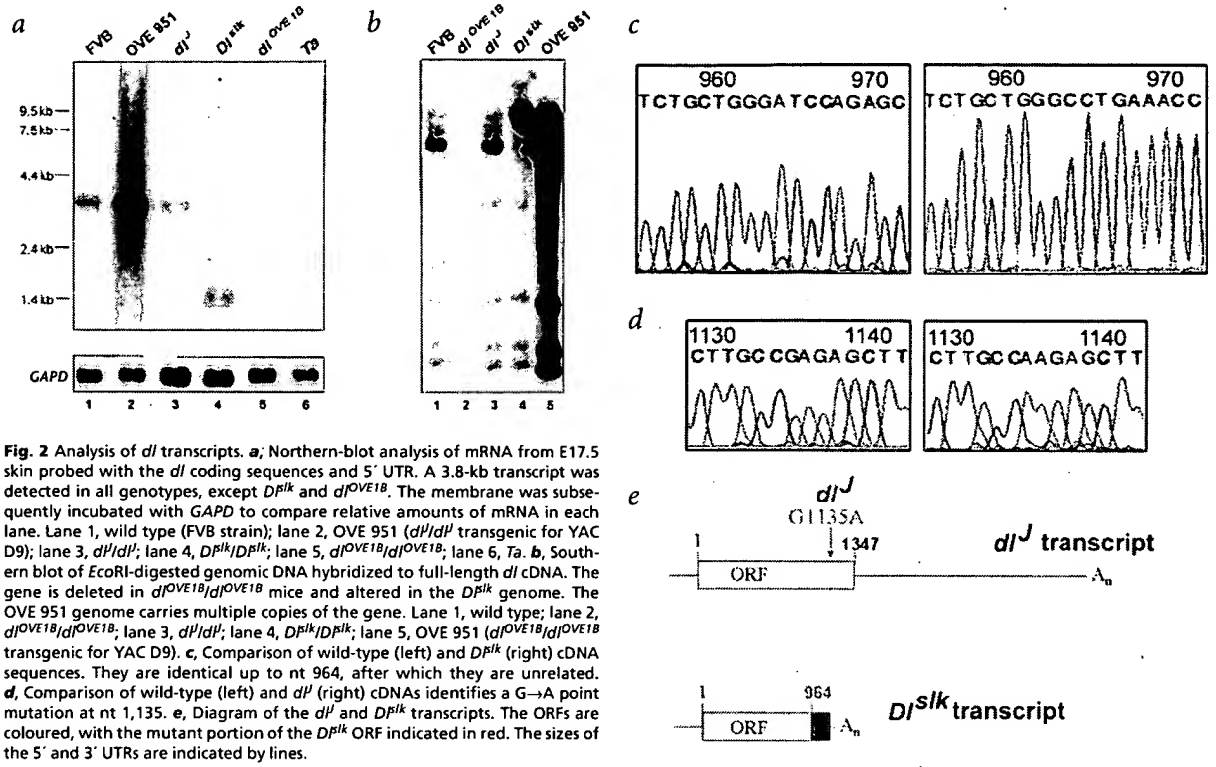

Fig. 2 Analysis of *dl* transcripts. ***a***; Northern-blot analysis of mRNA from E17.5 skin probed with the *dl* coding sequences and 5′ UTR. A 3.8-kb transcript was detected in all genotypes, except Dl^{slk} and dl^{OVE1B}. The membrane was subsequently incubated with *GAPD* to compare relative amounts of mRNA in each lane. Lane 1, wild type (FVB strain); lane 2, OVE 951 (dl^{J}/dl^{J} transgenic for YAC D9); lane 3, dl^{J}/dl^{J}; lane 4, Dl^{slk}/Dl^{slk}; lane 5, dl^{OVE1B}/dl^{OVE1B}; lane 6, *Ta*. ***b***, Southern blot of *Eco*RI-digested genomic DNA hybridized to full-length *dl* cDNA. The gene is deleted in dl^{OVE1B}/dl^{OVE1B} mice and altered in the Dl^{slk} genome. The OVE 951 genome carries multiple copies of the gene. Lane 1, wild type; lane 2, dl^{OVE1B}/dl^{OVE1B}; lane 3, dl^{J}/dl^{J}; lane 4, Dl^{slk}/Dl^{slk}; lane 5, OVE 951 (dl^{OVE1B}/dl^{OVE1B} transgenic for YAC D9). ***c***, Comparison of wild-type (left) and Dl^{slk} (right) cDNA sequences. They are identical up to nt 964, after which they are unrelated. ***d***, Comparison of wild-type (left) and dl^{J} (right) cDNAs identifies a G→A point mutation at nt 1,135. ***e***, Diagram of the dl^{J} and Dl^{slk} transcripts. The ORFs are coloured, with the mutant portion of the Dl^{slk} ORF indicated in red. The sizes of the 5′ and 3′ UTRs are indicated by lines.

is predicted to function as a cell surface receptor, we have called the protein Edar (for ectodermal dysplasia receptor). The mature Edar extracellular domain contains 14 cysteine residues, with only the 6 closest to the N terminus approximating the canonical Tnfr consensus. This is not unusual, as the transmembrane proximal cysteines of Tnfr family members frequently do not match the consensus[9]. Edar also contains a cytoplasmic region similar to the 'death domain' (Fig. 1*c*) of other Tnfr-like proteins. Although frequently involved in transducing apoptotic signals, death domains appear to be more generally involved in mediating protein-protein interactions[10].

We determined *dl* transcript sizes and expression levels in wild-type, mutant and YAC D9 transgenic family OVE951 (cured *dl* mutant) skin by northern-blot analysis (Fig. 2*a*). We also analysed each genotype by Southern-blot analysis (Fig. 2*b*). A 3.8-kb transcript was detected in wild-type, OVE 951, dl^{J} and *Ta* embryonic skin. The OVE 951 lane had a stronger hybridization signal, agreeing with Southern-blot data indicating a high copy number in this transgenic family (Fig. 2*b*). The dl^{OVE1B} deletion mutant gave no *dl* hybridization signal on either northern (Fig. 2*a*) or Southern (Fig. 2*b*) blots. Dl^{slk} mice synthesize a truncated transcript of 1.4 kb (Fig. 2*a*) because of a genomic alteration (Fig. 2*b*).

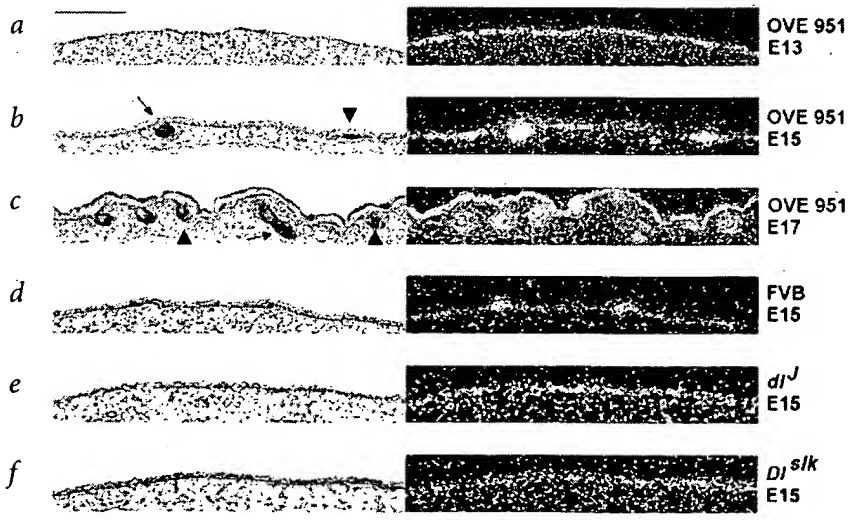

Fig. 3 Expression of *dl* in skin. Bright field (left) and corresponding dark field (right) images are shown for *in situ* hybridization using a ^{35}S-labelled riboprobe. E13 (*a*), E15 (*b*) and E17 (*c*) OVE 951 YAC transgenic skin and E15 wild-type (*d*), dl^{J} (*e*) and Dl^{slk} (*f*) skin are shown. ***a***, At E13, before hair follicle induction, *dl* is expressed throughout the basal layer of the epidermis. ***b***, By E15 expression has become upregulated in foci of induced cells initiating (arrowhead) and undergoing (arrow) follicular morphogenesis, and is beginning to be downregulated in surrounding cells. ***c***, By E17 *dl* is expressed in secondary follicles (arrowheads) and elongating primary follicles (arrow), but is no longer detected in the interfollicular epidermis. ***d***, The expression pattern in E15 wild-type skin is the same as in OVE 951, but transcript levels are lower. dl^{J} (***e***) and Dl^{slk} (***f***) mutant epidermis do not exhibit localized *dl* upregulation during the first wave of folliculogenesis. All images show dorsal skin. Scale bar, 200 μm.

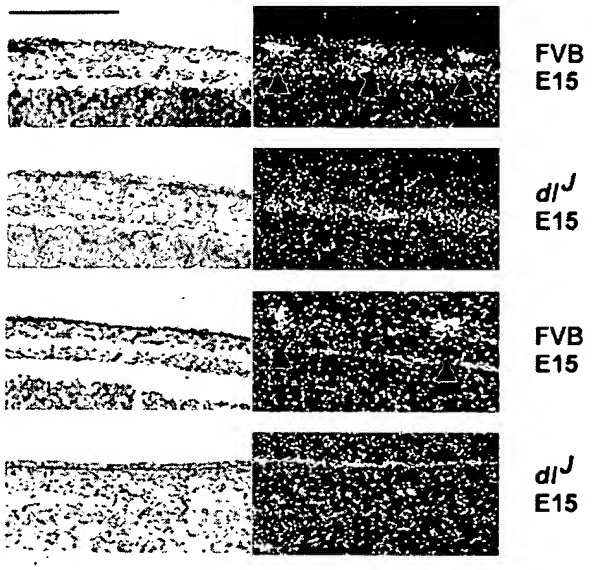

Fig. 4 Absence of expression of early hair follicle markers in *dl* mutant skin. Bright field (left) and corresponding dark field (right) images are shown for *in situ* hybridizations using ^{35}S-labelled riboprobes. ***a***, *Bmp4* expression in E15 wild-type dorsal skin is present in dermal cells underlying all newly induced placodes (arrowheads). ***b***, *Bmp4* is not expressed in dl^J dermis at E15. Expression of *Bmp4* in the layer of cutaneous muscle underlying the dermis serves as a positive control for hybridization. ***c***, *Shh* is focally expressed in the epidermis of all placodes in E15 wild-type dorsal skin (arrowheads). ***d***, *Shh* is not expressed in dl^J epidermis at this age. Scale bar, 200 μm.

Primers from the 3′ end of *dl* failed to generate an RT-PCR product from Dl^{slk} skin mRNA, suggesting that this part of the transcript is missing. We used 3′-RACE to amplify the 1.4-kb Dl^{slk} cDNA. The sequence of this cDNA matches that of wild type up to nt 964, after which the Dl^{slk} transcript contains a further 185 nt (Fig. 2*c*,*e*), the sequence of which is identical to the early transposon[11]. Thus the Dl^{slk} mutation was caused by integration of a transposon into the *dl* coding sequence. An ORF is maintained for 149 of these residues, and is followed by a 36-nt 3′ UTR that includes a polyadenylation signal. The protein encoded by Dl^{slk} is presumably capable of binding ligand and associating with wild-type receptor, but lacks the cytoplasmic sequences required for intracellular signal transduction.

We amplified the *dl* ORF from dl^J cDNA and directly sequenced it. We found it to be identical to wild type, except for a G→A point mutation at nt 1,135 (Fig. 2*d*,*e*), which we confirmed by amplifying and sequencing genomic DNA. This mutation results in a glutamate-to-lysine substitution at amino acid 379 within the predicted death domain (Fig. 1*a*,*c*). Although it is not clear why the dl^J mutation is recessive, whereas Dl^{slk} is dominant, the phenomenon of both dominant and recessive intracellular mutations has also been observed in Tnfr1 (ref. 12).

We examined the *dl* expression pattern in developing skin by *in situ* hybridization of sectioned mouse embryos. The expression pattern in OVE 951 skin is the same as in wild type, but the hybridization signals from the YAC transgenic mice are stronger (Fig. 3*b*,*d*). Before follicle initiation, *dl* transcripts are uniformly present in the basal cells of the epidermis (Fig. 3*a*). Expression becomes focally elevated before the appearance of morphologically identifiable placodes (Fig. 3*b*,*d*), and remains high in the follicle epithelial cells that retain contact with the dermal condensation at the base of the epidermal downgrowth (Fig. 3*b*,*c*). By E17, *dl* transcripts are almost exclusively confined to the maturing follicles and the recently initiated placodes, with reduced expression in the interfollicular epidermal cells (Fig. 3*c*). In contrast, dl^J and Dl^{slk} mutants lack any sign of placode formation on the trunk at E15 (Fig. 3*e*,*f*). They exhibit homogeneous *dl* expression (Fig. 3*e*,*f*), implying that *dl* upregulation is dependent on Edar activity in skin at this age.

We also examined expression of the pre-placode markers *Bmp4* and *Shh*[13,14] in wild-type and mutant skin at E15 to determine whether induction is initiated in the absence of Edar function. *Bmp4* is expressed in pre-placode clusters of mesenchymal cells in E15 wild-type skin (Fig. 4*a*), but not in *dl* mutant skin at this age (Fig. 4*b*). *Shh* is expressed in complementary clusters of epidermal cells in wild-type skin at the sites of placode initiation (Fig. 4*c*). *Shh* expression was not induced at E15 in mutant skin (Fig. 4*d*). These *in situ* hybridization data suggest that Edar signalling is required at the very earliest stages of hair follicle induction and may be responsible for receiving the initial hair follicle-inducing signal.

The *Ta* mutation causes a phenotype identical to that of *dl* mutants (ref. 1). *Ta* cDNA has been cloned and found to encode a novel type II transmembrane protein (called ectodysplasin, Eda) that contains a gly-X-Y repeat domain similar to that mediating trimerization of collagen subunits[15–17]. Sequence analysis revealed that the carboxy-terminal region of Eda is homologous to members of the Tnf family (data not shown), which are also type II membrane-associated proteins that form homotrimeric complexes[18]. On the basis of Eda being a type II membrane protein, its likely ability to trimerize, its similarity to Tnf and its mutant phenotype, we predict that Eda is the Edar ligand.

Widespread expression of *dl* in unspecified epidermis (Fig. 3*a*) suggests that a localized Edar ligand might act as the hair follicle-inducing signal. *Eda* is widely expressed at low levels in embryonic and adult epidermis, but with little or no dermal expression, and there is currently no indication of focal *Eda* upregulation during hair follicle induction[16,19]. Furthermore, skin recombination experiments indicate that the *Ta* gene prod-

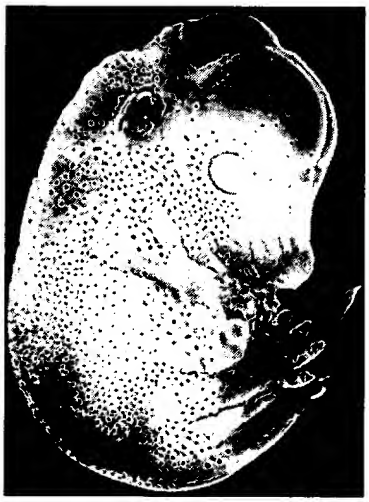

Fig. 5 Whole-mount *in situ* hybridization of *dl* to an E15 OVE 951 embryo. Placodes are visible as scattered dots, with low levels of expression in the surrounding cells, suggesting lateral inhibition of hair follicle fate. The tail and limbs have relatively high transcript levels throughout the as yet uninduced epidermis. Whisker follicles are too deep for probe penetration. Hybridization to the eyelid and joints in the toes can also be seen.

uct, now known to be Eda, is active over a distance greater than that separating hair follicles[20,21], suggesting that Eda may be released from the membrane by proteolytic cleavage, like other Tnf relatives[18]. If Eda is a diffusible ligand for Edar, then a mechanism to prevent ubiquitous hair follicle induction must exist. This may rely on either lateral inhibition of hair follicle fate in uninduced cells by nearby cells already committed to a placode fate, or a requirement for a locally restricted co-inducing signal.

A mechanism of lateral inhibition is suggested by the *dl* expression pattern. Whole-mount *in situ* hybridization (Fig. 5) shows that most placodes with upregulated *dl* expression are surrounded by a halo of cells with little or no expression. Commitment to a hair follicle fate may involve localized positive feedback through upregulation of *dl* expression, coupled with lateral inhibition of *dl* expression in adjacent epidermis to generate the hair follicle pattern.

Models for Edar function should take into account the fact that *dl* and *Ta* mice do generate a subset of hair follicles, beginning around E17. During normal mouse fetal development, hair follicles are initiated in temporally distinct waves[22,23]. The follicles that initiate in the first wave, between E14 and E16, fail to form in *dl* and *Ta* mice[23–25], but the second wave of folliculogenesis, beginning around E17 (ref. 22), does occur in these mutants[23,25]. This suggests that Eda and Edar are required specifically for primary follicle induction, with alternative pathways being responsible for the secondary hair follicles. The transcription factor Lef1 may be an essential factor in this putative second pathway. *Lef1*-null mutants have hair on the tail, eccrine sweat glands and some follicles on the trunk[26], thus exhibiting a phenotype reciprocal to that of *dl* or *Ta* mutants. This suggests that induction of primary follicles may be Edar dependent and Lef1 independent, whereas in secondary follicles this situation is reversed.

Although it is not clear why two separate inductive pathways exist, one reason may be the need to generate a complex insulating undercoat. In the first wave of folliculogenesis, primary follicles initiate at approximately equal distances from one another and appear to prevent other primary follicles from forming nearby (Fig. 5). A different molecular system for the second wave of follicle induction might evade inhibition by the existing primary follicles, allowing the development of a spatially complex fur coat.

We have identified here a cell surface receptor that is involved in inductive specification of hair follicle fate. It is notable that *dl* encodes a Tnfr relative, as other members of this family are primarily involved in maintenance of homeostasis and immune regulation[8], rather than cell fate specification during development. In addition, the mouse *dl* cDNA described here has been used to clone its human orthologue and mutations have been identified in dominant and recessive hypohidrotic ectodermal dysplasia families[27].

Methods

BAC library screening. Two sets of oligonucleotides were designed based on sequencing of YAC D9 subclones and were used to screen a pooled mouse BAC library by PCR (Human and Molecular Genetics Department, Baylor College of Medicine). The oligonucleotides were: 5′–ATCATGGCTGTGCACTCTAG–3′ (27209) and 5–ACCTACTGCATGTCTGTGGA–3′ (27210); 5′–CACATGCTCAGTGTTGTCCA–3′ (27213) and 5′–ACACAG GCTCAGTCATGCGG–3′ (27214).

cDNA selection. The cDNA selection procedure was as described[28]. The driver was a biotinylated BAC 508K21 PCR population, and we prepared the cDNA population for hybridization from pooled E13, E17 and P0 mouse skin, which we blocked with dl^{OVE1B}/dl^{OVE1B} genomic DNA before hybridization. cDNA was subjected to two rounds of driver enrichment. We cloned cDNA from the second enrichment into pT-Adv (Clontech).

cDNA library construction and screening. We isolated poly(A)$^+$ RNA from E17.5 OVE 951 (ref. 6) skin, foot and tail, and used it to prepare a cDNA library using the λ ZAP-cDNA synthesis kit (Stratagene). The library was plated and screened using [^{32}P]-dCTP labelled *cDS446*.

Nucleic acid preparation and hybridizations. For northern analysis, total RNA was prepared from E17.5 skin, foot and tail (RNA STAT 60, Tel-Test) and each RNA sample (300 μg) was poly(A) enriched (Messagemaker Kit, Gibco). We separated recovered RNA on a formaldehyde-agarose gel and transferred it to a nylon membrane (Zeta Probe, Bio-Rad). [^{32}P]-dCTP-labelled *dl* 5′ UTR and ORF was used to probe the blot in sodium phosphate (0.5 M), 7% SDS at 65 °C overnight. Four post-hybridization washes were performed in 0.1×SSC, 0.1% SDS at 65 °C for 20 min each. Human *GAPD* cDNA was purchased from Clontech. Genomic DNA for Southern analysis was isolated from tail tissue, digested with *Eco*RI, separated on an agarose gel and transferred to a nylon membrane (Zeta Probe, Bio-Rad). We carried out hybridization to the entire [^{32}P]-dCTP-labelled *dl* cDNA and subsequent washing under the conditions described for northern-blot analysis.

RACE and RT-PCR. Poly(A)$^+$ RNA was isolated from E17.5 skin of OVE 951 mice and used for 5′-RACE. First strand cDNA was synthesized using random primers (SuperScript II kit, Gibco), then 3′ dC tailed with terminal transferase (Gibco). PCR amplification was performed using oligonucleotides 5′–CCTGAGAGCTCTTTGTGAG–3′ (10S) and 5′–CGGGATCCTCGAGGGGGGGGGGGGGGH–3′ (anchored oligo dG; H, A/C/T). The cycling conditions used were: 94 °C 2 min (×1); 94 °C 30 s, 58 °C 45 s, 72 °C 2 min (×40); 72 °C 10 min (×1). We sequenced the PCR product using 5′–AAGCAGAGCTCCACAATC–3′ (28753). Dl^{slk} newborn skin cDNA was prepared for 3′-RACE by generating first strand cDNA using oligodTVN 5′–GGCCGCTCTGGACAGGATATGTTTTTTTTTTTTTTTTVN–3′ (V, A/C/G; N, A/C/T/G) as primer (SuperScript II kit, Gibco). PCR was performed on the first strand reaction product using the oligonucleotides 5′–GGAACAGTCAAGAGCGAGTT–3′ (5′F) and 5′–GCGGATCCAGGCCGCTCTGGACAGGATATG–3′ (oligo dT nested). We used the following conditions: 94 °C 2 min (×1); 94 °C 30 s, 58 °C 45 s, 72 °C 1.75 min (×36), 72 °C 15 min (×1). The PCR product was directly sequenced using oligonucleotide 5′–AGTGAGAATGATGCCTCC–3′ (28756). cDNA was prepared from dl^{J} newborn skin using the SuperScript II kit (Gibco). The oligonucleotides used to amplify the dl^{J} mutation were 28756 and 5′–GCCTTTGTTCAGTCATAGG–3′ (28762). The cycling conditions used were: 94 °C 2 min (×1), 94 °C 30 min; 58 °C 45 s, 72 °C 1.5 min (×34); 72 °C 15 min (×1). Oligonucleotide 28756 was used to directly sequence the RT-PCR product.

In situ hybridization. *In situ* hybridization to sectioned tissue was as described[29]. The entire *dl* cDNA was used to prepare an [^{35}S]-UTP-labelled antisense riboprobe. We used samples of dl^{OVE1B}/dl^{OVE1B} tissue as negative controls. We synthesized *Bmp4* riboprobe from a cDNA corresponding to nt 357–657 of the ORF. The *Shh* riboprobe was synthesized from full-length cDNA. Whole-mount *in situ* hybridization was performed as described with modifications for hybridization to the skin[30]. The digoxigenin-labelled riboprobe was transcribed from the entire *dl* 3′ UTR.

DNA sequencing. DNA sequencing was performed by either the Mental Retardation Research Center Sequencing Core Laboratory or by the Cell Biology Sequencing Core Laboratory at Baylor College of Medicine. PCR products were purified before sequencing using Qiagen PCR Purification or Qiaex II Gel Extraction kits.

Sequence analyses. BLAST database searches were performed using the National Center for Biotechnology Information web site (http://www.ncbi.nlm.nih.gov/). We identified the signal peptide by PSORT II (http://psort.nibb.ac.jp:8800/) and the transmembrane domain by the

Dense Alignment Surface method (http://www.biokemi.su.se/~server/DAS/). Sequence alignments were prepared using Clustal W (http://pbil.ibcp.fr/NPSA/npsa_clustalw.html) and shaded using the Boxshade program (http://www.isrec.isb-sib.ch:8080/software/BOX_form.html).

GenBank accession numbers. *dl* cDNA, AF160502; *Bmp4* cDNA, X56848; *Shh* cDNA, X76290; rat p75 Ngfr, P07174; mouse Fas, P25446; mouse Tnfr1, P25118.

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